Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of:

Inventor(s): **Vijay Mahant & Byron Doneen**

For (title): **Methods and Apparatus for Separation of Biological Fluids**

1. Type of Application

This application is a(n):

_X_ Non-Provisional (37 CFR §1.53(b))

_X_ Original

___ Continuation

___ Divisional

___ Continuation-in-part

___ Continued Prosecution Application (37 CFR § 1.53(d))

___ Continuation

___ Divisional

___ of parent application serial no. _____ filed _____ which claims priority to provisional application serial no._____ dated _____.

CERTIFICATE OF MAILING (37 CFR 1.8a)

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as Express Mail , with Express Mailing Label No. EL228561584US, in an envelope addressed to the Commissioner of Patents and Trademarks, Box New Application, Washington, DC 20231.

Collene Houston Dated: 3/2/99

Collene Houston

2. **Benefit of Prior U.S. Application(s) (35 USC 120)**

X None

___ of co-pending parent application referenced above.

___ of co-pending provisional application serial no. _____, filed _____.

3. **Filing Papers**

X Papers Enclosed Which Are

15 Pages of Specification

2 Pages of Claims

1 Pages of Abstract

2 Sheets of drawing

___ formal

X informal

___ in triplicate

___ Enclosed is a copy of the latest inventor-signed prior application, including a copy of the oath or declaration showing the original signature or an indication it was signed. I hereby verify that the papers are a true copy of the latest signed prior application number _____, and further that all statements made herein of my own knowledge are true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

___ Please use all the contents of the prior application file wrapper, including the drawings (if any), as the basis papers for the new application.

___ Please amend the new application by inserting the following sentence at the beginning of the specification:

This application is a ___ continuation ___ continuation-in-part ___ divisional of co-pending application serial no. _____ filed _____, (status, abandoned, pending etc.)

___ Please abandon the parent application at a time when the parent application is pending or when the petition for extension of time or to revive in that application is granted and when this application is granted a filing date so as to make this application copending with said parent application. At the same time please append the words "now abandoned" to the amendment to the specification set forth above.

4. Declaration or oath and power of attorney

X Enclosed

executed by (check **all** applicable boxes)

X inventor(s).

___ legal representative of inventor(s). 37 CFR 1.42 or 1.43

___ joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.

___ this is the petition by 37 CFR 1.47 and the statement required by 37 CFR 1.47 is also attached. See item 13 below for fee.

___ Not enclosed.

___ Application is made by a person authorized under 37 CFR 1.41(c) on behalf of all the above-named inventor(s). The declaration and oath, along with the surcharge required by 37 CFR 1.16(e) will be filed subsequently.

___ Is contained in the parent application.

5. **Additional papers enclosed**

X An assignment of the invention to Qualisys Diagnostics, Inc.

X is attached. X Assignment recordal attached.

___ was filed on _____, copy attached.

___ Preliminary Amendment

___ Information Disclosure Statement

___ Form PTO-1449

X Small Entity Statement

___ Declaration of Biological Deposit

___ Authorization of Attorney(s) to Accept and Follow Instructions from Representative

___ Special Comments

___ Other

6. **Inventorship Statement**

The inventorship for all the claims in this application are:

X the same

___ not the same. An explanation, including the ownership of the various claims at the time the **last** claimed invention was made,

___ is submitted.

___ will be submitted.

7. **Language**

X English

___ non-English

___ the attached translation is a verified translation. 37 CFR 1.52(d).

8. Certified Copy

Certified copy(ies) of application(s)

_____

(country) (appln. no.) (filed)

_____

(country) (appln. no.) (filed)

from which priority is claimed

___ is(are) attached

___ will follow.

9. Request for International-Type Search (37 CFR 1.104(d))

___ Please prepare an international-type search report.

10. Fee Calculation

X	Small Entity. Verified Statement(s) that this is a filing by a small entity under 37 CFR 1.9 and 1.27 is(are) either attached or already on file.

Basic Fees	Filed	Base	Extra	Rate Large	Rate Small	Fee
Utility Patent				$760.00	$380.00	$ 380.00
Total Claims	21	20	1	$18.00	$9.00	$ 9.00
Indep. Claims	1	3	0	$78.00	$39.00	$ .00
Multiple Dependent Claims	Yes			$260.00	$130.00	$ 130.00
Provisional Patent				$150.00	$75.00	$ .00
Design Patent				$310.00	$155.00	$ .00
Subtotal						$ 519.00

Extension Fees		Large Entity	Small Entity	Fee
-	one month extension	$110.00	$55.00	$ .00
-	two month extension	$380.00	$190.00	$ .00
-	three month extension	$870.00	$435.00	$ .00
-	four month extension	$1,360.00	$680.00	$ .00
Subtotal				$ .00
Miscellaneous Fees			Rate	Fee
-	recording assignment (37 CFR 1.21(h)(1))		$40.00	$ 40.00
-	fee for international-type search report (37 CFR 1.21(e))		$40.00	$ .00
-	fee for petition to make application special		$130.00	$ .00
Subtotal Miscellaneous				$ 40.00
Totals				
Subtotal Basic Filing Fees From Above				$ 519.00
Subtotal Extension Fees				$ .00
Subtotal Miscellaneous				$ 40.00
Total Fees				$ 559.00

11.Fee Payment Being Made At this Time

X Enclosed

___ Not Enclosed. No filing fee is to be paid at this time. (This and the surcharge required by 37 CFR 1.16(e) can be paid subsequently.)

12.Method of Payment of Fees

X Check no. 7294 in the amount of $559.00

___ Charge Account No. 500-341 in the amount of $ .00.

13. Authorization to Charge Additional Fees

X The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No.500-341

x 37 CFR 1.16(a) or (g) (filing fees)
x 37 CFR 1.16(b), (c) and (d) (presentation of extra claims)
x 37 CFR 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)
x 37 CFR 1.17 (application processing fees)
x 37 CFR 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 CFR 1.311(b))

14. Instructions As To Overpayment

X credit Account No 500-341.
___ refund

CROCKETT & FISH

Dated: February 25, 1999

By _____
ROBERT D. FISH
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PTO/SB/10 (12-97)
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STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) & 1.27(c))—SMALL BUSINESS CONCERN

Docket Number (Optional): 560.02-US1

Applicant, Patentee, or Identifier: Vijay K. Mahant and Byron A. Doneen
Application or Patent No.:
Filed or Issued:
Title: Methods and Apparatus for Separation of Biological Fluids

I hereby state that I am
- ☐ the owner of the small business concern identified below:
- ☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF SMALL BUSINESS CONCERN: Qualisys Diagnostics, Inc.

ADDRESS OF SMALL BUSINESS CONCERN: 16 Technology Drive, Suite 118, Irvine, CA 92618

I hereby state that the above identified small business concern qualifies as a small business concern as defined in 13 CFR Part 121 for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time, or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby state that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention described in:

- ☒ the specification filed herewith with title as listed above.
- ☐ the application identified above.
- ☐ the patent identified above.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights in the invention must file separate statements as to their status as small entities, and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern, or organization having any rights in the invention is listed below:
- ☒ no such person, concern, or organization exists.
- ☐ each such person, concern, or organization is listed below.

Separate statements are required from each named person, concern or organization having rights to the invention stating their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

NAME OF PERSON SIGNING: VIJAY K. MAHANT

TITLE OF PERSON IF OTHER THAN OWNER: VICE PRESIDENT, R&D

ADDRESS OF PERSON SIGNING: 16 Technology Drive, Suite 118, Irvine, CA 92618

SIGNATURE: V. K. Mahant DATE: 3/1/99

METHODS AND APPARATUS FOR SEPARATION OF BIOLOGICAL FLUIDS

FIELD OF THE INVENTION

The field of the invention is clinical diagnostics and biotechnology.

BACKGROUND OF THE INVENTION

In vitro diagnostic tests to identify and treat diseases have become common tools in hospitals, homes and physician's offices. Biological fluids such as blood, urine or cerebrospinal fluids, which may at times contain blood, are the most frequently employed biological samples for such tests.

Blood contains many different components, some of which are present in strikingly varied concentrations from sample to sample. The percentages of both red and white blood cells in whole blood, for example, can vary among normal individuals, and even in the same individual over time, and in particular under pathological conditions. This large variation coupled with other factors such as storage conditions, coagulation, and the fragility of red blood cells, produces considerable technical problems in performing diagnostics using blood-containing samples.

Whole blood is usually separated into various fractions prior to testing. Among other things, separation into fractions can advantageously compensate for differences in hematocrit values, and in other ways reduce potential interference in up stream or down stream biochemical assays. Frequently employed fractions are serum, plasma, white cells, red blood cells and platelets. The term "plasma" is used herein to mean any fluid derived from whole blood from which a substantial portion of the cellular components has been removed.

Blood separation technologies can be conceptually grouped into three categories - centrifugation, filtration, and separation.

Centrifugation

Blood separation is routinely achieved by centrifugation. Centrifugation is generally desirable because: (1) centrifugation can generally separate cellular components from serum or

plasma at an efficiency of greater than 95%; (2) centrifuges do not require highly trained personnel to operate; and (3) centrifugation allows concurrent processing of multiple samples in under 15 minutes. Centrifugation of blood is, however, also problematic. For example, centrifuges are expensive, involve multiple steps, are often unavailable at points of care such as bedside, schools or at home, and usually require electrical power for operation. Centrifugation also generally requires at least several milliliters of blood.

Filtration

Many filtration techniques are known for separating various components from blood. U.S. Pat. No.4,987,085 to Allen et al., for example, describes a filtering system with descending pore size using a combination of glass fiber membranes and cellulose membranes. U.S. Pat. No. 4,753,776 to Hillman et al. discloses a glass microfiber filter using capillary force to retard the flow of cells. U.S. Pat. No. 4,256,693 to Kondo et al. discloses a multilayered chemical analysis element with filter layers made from at least one component selected from paper, nonwoven fabric, sheet-like filter material composed of powders or fibers such as man-made fibers or glass fibers. U.S. Pat. Nos. 3,663,374 and 4,246,693 disclose membrane filters for separating plasma from whole blood and U.S. Pat. Nos. 3,092,465, 3,630,957, 3,663,374, 4,246,693, 4,246,107, 2;330,410 disclose further filtration systems, some of which make use of small-pore membranes.

Known filtration techniques generally reduce the volume of blood required to only a few drops. Many filtration tests therefore contemplate using only about 25 to 75 µl of whole blood. Some filtration techniques have even been developed that require only about 5 to 50 µl of whole blood. In most applications, filtration occurs directly on a test-strip in which the filtration surface is placed above the reaction zone or zones of the strip. Filtration in these formats also reduces or eliminates the availability problems associated with centrifuges.

But these advances often create entirely new problems. For example, filters tend to retain significant amounts of plasma, and analytes present in low concentrations are frequently difficult to detect in the serum derived from small volumes of blood. Existing filters also tend to clog, and have undesirably slow flow rates. Agglutinating agents are often mixed with whole blood to

reduce clogging and to improve flow rates, (see U.S. Pat. Nos. 5,262,067, 5,766,552, 5,660,798 and 5,652,148), but these problems remain.

Efforts have been made to improve the flow rate by modifying the force employed against the filter. But choices here are fairly limited. Filters are relatively simple to produce and use, but tend to cause excessive hemolysis of red blood cells. Capillary action, a phenomenon in which water or liquid will rise above normal liquid level as a result of attraction of molecules in liquid for each other and for the walls of a capillary can also be used. Capillary action, however, is generally too weak to effect rapid separation of large volumes. (See, for example, U.S. Pat. Nos. 5,660,798, 5,652,148 and 5,262,067). Moreover, separation of plasma by capillary action tends to retain a relatively large amount of fluid within the wicking membrane, or a collection membrane. This in turn may necessitate testing the wicking membrane or the collection membrane or both, or eluting the retained material from the membranes.

Solid-Phase Separation

Solid-phase separation typically involves a surface having binding to a target, the surface acting to immobilize and remove the target from a sample. Exemplary solid-phase separation techniques are binding chromatography, binding separation using beads, and hollow fibers separations.

One particularly advantageous type of solid-phase separation is magnetic separation, in which a target is captured by magnetically attractable (paramagnetic) beads. Since no physical barriers are present, as would be the case with filtration separation, magnetic separation tends to be relatively gentle. In U.S. Pat. No. 5,514,340 to Lansdorp and U.S. Pat. No. 5,123,901 to Carew, for example, magnetic wires are employed in batch processes to separate magnetic particles from a fluid. In U.S. Pat. No. 4,663,029 to Kelland et al. and U.S. Pat. No. 5,795,470 to Wang, magnetic particles are separated out from a fluid in a continuous flow process. Still other methods published for example in U.S. Pat. No.5,536,475 to Moubayed, employ rocking separation chambers and multiple magnets to separate magnetic particles from a fluid.

One of the major limitations of applying known magnetic separation to blood separation is that multiple anti-ligands are required to remove all of the various types of cells and sub-

cellular particles. Red blood cells, lymphocytes, monocytes, and platelets, for example, have different surface antigens, and do not specifically bind to any one antibody. Furthermore, lack or absence of ligands on the cells due to pathological conditions, genetic diseases or genetic variations or life cycle of cells generally reduce the efficiency with which the anti-ligands bind with the target cells.

The problems with known magnetic separation devices are exacerbated with increasing sample volumes, especially sample volumes over one milliliter. Since many diagnostic applications require serum volumes of up to one milliliter to satisfy the requirements of multiple tests or batteries of tests, magnetic separation has not been particularly useful. Moreover, assays such as glucose or hemoglobin tests are highly susceptible to interference caused by biological or chemical substances in the sample, including proteins, bilirubin, and drugs.

Thus, there is still a need to provide improved methods and apparatus for separating blood into its constituent parts, and especially for separating plasma from whole blood.

SUMMARY OF THE INVENTION

In accordance with the present invention, a cell containing sample is separated into a cell containing portion and a substantially cell depleted portion, by mixing the sample with both an additive and particles to produce a cell containing network, and separating the network from the remaining substantially cell depleted portion using a magnetic force.

In one aspect of preferred embodiments the sample comprises whole blood, and the cell-containing portion largely comprises a network of inter-linked red blood cells. Especially preferred linkers include anti-ligands such as primary antibodies that bind to a ligand or an antigen on or in red blood cell membranes, and secondary antibodies that bind to the primary antibodies. In another aspect of preferred embodiments, the primary antibodies are added directly to the sample, and the secondary antibodies are coupled to the surfaces of paramagnetic beads.

In another aspect of preferred embodiments, polymeric materials such as Polybrene®, cationic liposomes, cationic lipids, and polydendromers may be used in combination with anti-ligand(s) and magnetic separation or in combination with anti-ligand(s) and filtration.

In yet another aspect of preferred embodiments, aptamers can be used as anti-ligand(s) by themselves or in combination with cationic polymers, cationic liposomes, and dendromers.

Various objects, features, aspects and advantages of the present invention will become more apparent from the following detailed description of preferred embodiments of the invention, along with the accompanying drawings in which like numerals represent like components.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic of a preferred embodiment in which separating agents are being added to a blood sample.

Figure 1B is a schematic of the embodiment of figure 1A following separation of red blood cells.

Figure 2A is a schematic of an alternative embodiment.

Figure 2B is a schematic of binding interactions contemplated to be present in the embodiment of Figure 2A.

Figure 3 is a schematic of another alternative embodiment.

DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS

In the generalized preferred embodiment of **Figure 1A**, a blood separation apparatus 10 comprises a vessel 20 and a magnet 50. The vessel 20 contains blood 30, to which is being added primary antibodies 42 having a substantial binding to red blood cells, and particles 40 coated with a secondary antibody having a substantial binding to primary antibodies 42.

Vessel 20 is preferably an ordinary test tube or test tube-like vessel such as a vacutainer or falcon tube. The volume of such tubes is preferably less than about 10 ml, although it is contemplated that appropriate vessels may define sample cavities of greater or lesser volumes.

Although vessel 20 is depicted as having a typical test-tube shape, alternative vessels are contemplated to have different shapes. Thus, suitable vessels may have a narrowed top portion to

facilitate recovery of the substantially red blood cell depleted portion. Alternative vessels may also have differently shaped bottoms, such as a V-shaped or a flat bottom. In yet another example, an individual vessel may be formed as part of an array, such as in a multi-well microtiter plate. Further examples of suitable vessels include, hollow fibers, arrays of capillaries, beakers, pouches, dishes and cylinders – generally any device that can retain fluid within confining walls, and provide at least one opening. Appropriate vessels are even contemplated to include "open walled" structures such as a microscope slide having microchannels etched on glass or plastic, or a simple plastic foil or film. It is specifically contemplated that vessels employed in conjunction with the teachings herein may have at least one flexible wall.

It is contemplated that vessels can be made from any appropriate material or materials, including glass, synthetic polymers, ceramics, metals, or mixtures thereof. Such vessel can be colored or transparent, translucent or non-translucent, and may or may not have graduation or other markings.

In Figure 1A the sample being separated is whole blood. Such blood is generally contemplated to be fresh human whole blood, a few milliliters of which are preferably obtained by venipuncture. Another example is capillary blood, which can be obtained in volumes ranging from less than 10 to hundreds of μLs by use of a lancet.

The blood can be pre-treated, such as by addition of an additive, or removal of a component. Contemplated additives include buffer, water or isotonic solution, anticoagulants, antibodies, and test solutions. Contemplated substances or components that can be removed include antibodies, globulins, albumin, and cellular fractions such as platelets, white blood cells etc.

The blood can also be derived from non-human sources, including vertebrate or invertebrate animals. Blood employed as set forth herein can also be taken from any type of storage, and as such may be cooled blood, frozen blood, or blood with preservatives.

In preferred embodiments, the primary antibodies 42 are mouse derived monoclonal antibodies to human red blood cells, which in the field would often be referred to as monoclonal

Ab to hRBCs. The secondary antibodies 44 are preferably sheep derived anti-mouse IgG antibodies.

Techniques for raising the antibodies are well known. For example, both the primary and secondary antibodies can be derived from any appropriate source including, goat, sheep, horse or recombinant sources. Suitable antibodies can also be selected from many classes and subclasses, including IgG and IgM, and subclasses. Furthermore, antibodies can be selected from numerous molecular varieties, including proteolytic fragments or engineered fragments such as Fab or $(Fab)_2$, or chimeric antibodies. Combinations of antibodies are specifically contemplated.

Of course, both primary and secondary antibodies would advantageously have substantial binding to their respective targets. The primary antibodies would preferably have substantial binding to red blood cells, and in particular would have substantial binding to at least one ligand or component present on a surface of the red blood cells. The secondary antibodies would preferably have substantial binding to at least some component of the primary antibodies.

The secondary antibodies 44 are preferably included in the coating of coated particles 40. Such particles are attractable by magnetic force, and preferably comprise a paramagnetic composition embedded in synthetic polymers or cellulose. Although paramagnetic particles are preferred, the coated particles can also or alternatively include ferromagnetic or chromium material or mixtures thereof. In still further variations, suitable particles can be coated with many other materials including natural or synthetic polymers, agarose etc. The preferred particle size is in the range of 0.1-100μm, but alternative sizes between 10-100 nm or larger than 100μm are also contemplated. Viewed from another aspect, it is contemplated to employ particles having a mean volume between about 5×10^{-24} m^3 and about 5×10^{-6} m^3. Where red blood cells are being targeted, the diameter of the red cells may advantageously be about five times the diameter of the coated particles.

The term "coated" is used herein to mean any complete or partial covering of any exposed surface. In Figure 1, the particles 40 are coated with a material that immobilizes the secondary antibodies. Such immobilization can be temporary or permanent, and can involve covalent or non-covalent binding. For example, non-covalent binding may involve incubating

antibodies with the bead or other solid-phase. As another example, covalent coupling of antibodies to a solid-phase may involve including reacting amino groups of an antibody with aldehydes on the solid-phase, or activated carboxyl groups on the solid-phase, resulting in a covalent bond.

In yet other embodiments, one or both of the primary and secondary antibodies can be replaced by or complimented with an alternative composition have the desired binding, and at least a minimally acceptable specificity. Anti-ligands are a general class of such alternative compositions, and are defined herein as any molecule that binds non-covalently to an appropriate ligand. Examples of anti-ligands and ligands include and are not limited to antibodies and antigens, respectively, and sense and anti-sense oligonucleotides in nucleic acids. Other polymers are contemplated as well as nucleotides. Additional examples are aptamers and lectins having a substantial binding to ligands.

The term "a cell containing network" refers herein to an aggregate of at least more than one cell, from which individual cells cannot readily be mechanically removed without lysing the removed cells. Normally clotted blood is one example of a cell containing network, but aggregates formed substantially by any combination mediated by molecular interactions such as hydrophobic-, hydrophilic-, electrostatic-, van-der-Waals-, ionic interaction or other molecular interactions are also contemplated. Thus, other examples of cell containing networks are aggregates of red, white or other cells formed by combinations with antibodies or other linking agents having substantial binding to the cells. It is especially contemplated that such networks may include solid supports such as beads.

It is especially contemplated that heterogeneous aggregates can be formed using a mixture of red blood cells with two different antibodies, wherein the primary antibody binds the red blood cells, and the secondary antibody binds to the primary antibody. If only one of the two binding portions of the antibody is involved in such binding, the following aggregates can be formed: (a) primary antibody bound to a red blood cell; (b) secondary antibody bound to the a primary antibody only; and (c) secondary antibody bound to primary antibody that is bound to a red blood cell or some other cell type in blood or body fluids. If both of the two binding portions

of the antibodies are involved in binding, any combination of the aggregates (a), (b), (c) may be formed, thereby producing a potentially vast network of aggregates.

The sample, antibodies or other anti-ligands, and beads or other particles may be combined in the vessel in any order. For example, in one class of embodiments (not shown), vessels are contemplated to be pre-loaded with magnetically attractable beads. Suitable such vessels are commercially available as MiniMACS™ separation columns from Miltenyi Biotec™, and the columns are even provided with a separation enhancing device. The standard protocol would need to be modified to conform to the teachings herein, such as by pre-coating the beads with an appropriate anti-ligand, and by adding an appropriate anti-ligand to the sample.

The magnet 50 is generally a disc magnet, but in alternative embodiments the magnet can also have different shapes and designs. Contemplated alternative magnets include bar magnets, horseshoe magnets, ring magnets, and can have any suitable multiple pole geometry including quadrapoles, hexapoles and octapoles, etc. Magnets can be of the permanent type, electro-magnets, or even super-conducting magnets, and may comprise ferromagnetic or rare earth magnets. Furthermore, the magnet need not be a single magnet, but can advantageously comprise a plurality of magnets. Preferred magnets have strengths in the range of 0 to 2 Tesla for permanent magnets, or 0-100 Tesla for electromagnets. Especially preferred magnets employ a permanent magnet of field strength 0 to 1 Tesla.

In **Figure 1B** several networks 45 have been formed from the blood cells 32 (not shown in detail), antibody coated paramagnetic particles 40 (not shown in detail), and anti-red blood cell antibodies 42 (not shown in detail). The particles 40 within the network 45 are being attracted by magnet 50, thereby separating out the cell-containing networks 45 from the substantially cell-depleted plasma 34.

Red blood cells 32 are generally mature non-nucleated erythrocytes. These blood cells usually are the predominant form of red blood cells present in a sample. In alternative embodiments, red blood cells can also be red blood cells carrying any type of hemoglobin including α, β, γ or fetal hemoglobin. The red blood cells can also be regular healthy blood cells or red blood cells giving raise to diseases e.g. sickle cell anemia or thalassemia. Appropriate red

blood cells can also be in many stages of development, e.g. nucleated erythroblasts or aged, non-nucleated erythrocytes.

The inventive subject matter is, of course, not limited to mature non-nucleated erythrocytes, and specifically contemplates other stages of red cell development, other cells including white cells, and even cellular fragments including platelets. Thus, for example, where the sample comprises urine, a clinician or other individual may employ the inventive methods and apparatus to separate out bacterial cells or sloughed off bladder or urethral cells, and in such instances the red cells 32 of Figure 1B may be replaced with non-erythrocytes.

In addition to operating on a wide variety of samples, it is also contemplated that the inventive methods and apparatus described herein can be employed to measure a wide variety of analytes. Contemplated analytes include tumor markers such as prostate specific antigen (PSA), infectious disease markers, endocrine markers such as testosterone, estrogen, progesterone and various cytokines, and metabolic markers such as creatinine, glucose.

In **Figure 2A** a blood separation apparatus 110 has a soft-walled or otherwise flexible vessel 120 containing a network 145 formed from whole blood 130 comprising red blood cells 132 (not shown in detail), anti-red blood cell antibodies 142 (not shown in detail)and anti-mouse antibodies 144 (not shown in detail). A filter 160 filters out the network 145, and allows plasma 134 to pass through to a collection area.

The filter 160 is preferably a glass fiber filter having a pore size below the size of the cellular components of blood or larger than the individual cells, but smaller than the network. In alternative embodiments the filter can be made from many materials including chromatographic paper, natural or synthetic fibers, porous membranes etc. Examples for those alternative filters are nylon fiber filters, size exclusion membranes, paper filters, woven fabric filters. Furthermore, the filters may or may not be coated with material e.g. to reduce hemolysis or to specifically retain selected fractions or molecules. Appropriate coatings include polyvinylalcohol, polyvinylacetate, polycationic polymers, lectins or antibodies.

In Figure 2A the filtrate portion of the sample is passed through the filter by gravity. However, it is recognized that the driving force to move the sample through the filter can be a

force or pressure differential across the membrane, and can be achieved in many ways including centrifugation, vacuum, compressed gas, or a magnet as described elsewhere herein. The filtration time can vary greatly, but is generally considered to be within the range of a few seconds to less than 30 min.

Figure 2B depicts details of a possible portion of network 145, which includes anti-red blood cell antibodies 142 bound to red blood cells 132, and anti-mouse antibodies 144 bound to anti-red blood cell antibodies 142. Those skilled in the art will recognize that a single network can contain millions of cells, and it should be appreciated that the orientation and connections of the various components in Figure 2B are purely exemplary, and would not necessarily ever be found in an actual network. Among other things, a real-life network would be three-dimensional, rather than the two dimensional schematic as shown, and the antibodies would be much smaller than that shown in the drawing. It should also be appreciated that network 45 of Figure 1B is contemplated to have corresponding structures to that depicted in Figure 2B.

In **Figure 3** a blood separation apparatus 210 has a vessel 220 that receives a blood sample 230, a pre-filter 260 coated with anti-red blood cell antibodies 264, and a secondary filter 270 coated with anti-red blood cell antibodies 274. A portion of the sample 230 has filtered through the pre-filter 260 to provide a partially cell depleted fluid 233, and a portion of the cell depleted fluid 233 has filtered through the secondary filter 270 to provide a substantially cell depleted fluid 234.

Here, the vessel 220 is contemplated to be a vessel falling within bounds of vessels previously described with respect to vessel 20, and similar correspondences exist with respect to blood 230 and 30, red blood cells 232 and 32, primary antibodies 264 and 42, secondary antibodies 274 and 44, and plasma 234 and 34.

The preferred pre-filter material is nylon wool 260, comprising an uncompressed layer of nylon fibers. The secondary filter 270 is preferably a glass fiber disk onto which mouse anti-red blood cell antibodies 274 are bound. In alternative embodiments, either or both of the filters 260 and 270 can be substituted with any other suitable filter material including a fibrous filter material, filter paper, porous membranes etc. Examples hereof include coated or uncoated glass

fibers, mineral wool, chromatographic paper etc. Furthermore, the filter material may or may not be coated e.g. to reduce hemolysis or to specifically retain selected fractions or molecules. Appropriate coatings include polyvinylalcohol, polyvinylacetate, polycationic polymers, lectins or antibodies other than that previously described.

Experiments

Tests to separate red blood cells from plasma were performed, and the results are described below. These tests are only intended to be illustrative of some of the principles set forth above, and are not intended to be read as limitations on the scope of the claimed subject matter.

Experiment Set 1

In a first series of experiments, precipitation of red blood cells was performed using mouse anti-red blood cell antibodies, paramagnetic beads coated with goat anti-mouse antibodies and 0.5 ml of anti-coagulated whole blood. Heparinized, EDTA or citrated whole blood was mixed with 10 µl undiluted mouse anti-red blood cell antibody solution (Red Out™; Robbins Scientific Corp.) for 2 minutes; next 10 µl of a solution (isotonic PBS, pH 7.4) containing paramagnetic beads (Cortex Biochem Inc., MagaCell™ or MagaBeads™; 30 mg/mL; or Pierce MagnaBind™) coated with goat anti-mouse antibodies was added and mixed gently for 2 minutes. The bottom of the tube was placed on a magnet (permanent iron magnet; approximately 0.2 Tesla). Precipitation of the cellular network started instantly, and was substantially finished after about 2 minutes. Plasma was collected by aspiration from the top of the vessel.

Significantly, methods and apparatus described herein have been found to separate at least 70% (by volume) of the theoretically available cell depleted portion from the network within a relatively short period of time. In many cases the time period for such 90% separation is less than 30 minutes, in other cases less than ten minutes, and in still other cases less than 2 minutes. Separations have also been performed using the methods described herein that achieve at least 80%, at least 90%, at least 95% and at least 98% of the theoretically available cell depleted portion from the network within less than 30 minutes.

Experiment Set 2

In a second series of experiments, precipitation of red blood cells on a microscope slide was performed using whole blood, mouse anti-red blood cell antibodies and paramagnetic beads coated with goat anti-mouse antibodies. To 0.2 ml fresh whole blood, 5 µl of an anti-red blood cell antibody containing solution (Red Out™) was added, mixed and incubated for 2 min at room temperature. Then, 5 µL of a solution containing paramagnetic beads coated with goat anti-mouse antibodies (MagaCell™ or MagaBeads™ or MagaBind™) was added, mixed, and after another 2 minutes, two disk magnets were positioned at opposite ends of the slide. After about 1.5 minutes, a clear plasma containing zone was formed between the two magnets and this was retrieved with a pipette without disturbing the laterally-fixed cell containing network.

Experiment Set 3

Flat envelopes sealed on three of four sides were prepared from transparency acetate sheets (for example, the 3M Inc. product, 3MCG3460), or plastic sheet protectors (e.g., Avery-Dennison PV119E), or from small "ZipLock" or ITW Inc. MiniGrip™ bags (2.5 X 3 cm; 2.0 mil). One-half to 1 mL of anti-coagulated whole blood was injected into the bag; 10 uL of Red Out™ (see above) was added and mixed with blood by gently rocking the container. After 2 minutes, the anti-mouse coated magnetic beads (MagaCell™) were added, mixed and the open side of the bag sealed. Two minutes later the bag was placed horizontally on a rectangular permanent iron magnet (approximately 0.2 Tesla). The magnetic particles and attached cellular networked moved adjacent to the magnet, leaving a clear layer of plasma as supernatant. The bag was then rotated into an upright position while still on the magnet, opened, and plasma aspirated using a pipette. It was also discovered that envelopes or bags (containing blood previously treated with anti-RBC antibodies and anti-mouse coated paramagnetic beads) could be passed between two permanent magnets separated a narrow distance. As the sack was drawn upward between magnets, the cellular network was pulled to the bottom of the container, producing an overlying layer of plasma.

Experiment Set 4

In one experiment, a small amount of steel wool (washed and treated with 5 mg/mL BSA in PBS for 12 hours) was added to the sample container prior to addition of blood and precipitating reagents. After addition of RedOut™ and MagaCell™ reagents (each for 2 minutes), the tube was placed on the magnet. The paramagnetic beads contained within the cellular network were immobilized to the steel wool. A pipette tip was used for aspiration of plasma that was essentially free of blood cells. When coated with protein or some other polymer, the steel wool caused very little hemolysis in the cellular pellet in a 2-hr period. It is further contemplated that iron wire, wool, or beads could be added as above if it were coated with other non-hemolyzing polymers such as dextran, polyvinylpyrilidone on polyethylene glycol etc.

In another experiment, 15 iron brads were taped around the external bottom 1/3 of a 12 mm glass tube in which whole blood was incubated as described above with mouse anti-red blood cell antibodies and paramagnetic beads coated with goat anti-mouse antibodies. Placing the tube on a rectangular magnet produced an almost immediate deposition on the bottom and sides of the tube. The external brads place the magnetic source closer to the sample tube, thus applying a relatively uniform source of secondary lateral attraction to the entire sample column. As the cellular network moves to the sides of the tube, it aggregates further.

Experiment Set 5

Test results produced in accordance with methods and apparatus described herein are depicted in Table 1. In this regard it should be noted that centrifugation is at least 99% effective in removing cellular matter from whole blood, (99% separation efficiency) and that methods and apparatus described herein (listed as "Device" in the table) are almost as effective. In particular, methods and apparatus described herein can be described as having separation efficiency of least 90%, at least 95%, and at least 98%.

Table 1

Subject	Plasma Volume mL Centrifugation	Device	PSA Ng/mL Centrifugation	Device	Testosterone ng/mL Centrifugation	Device	Creatinine mg/dL Centrifugation	Device
1	0.45	0.36	28.5	27.2	4.78	5.45	0.75	0.64
2	0.55	0.48	25.1	23.2	14.45	11.2	0.70	0.62
3	0.53	0.48	27.2	26.4	16.19	15.18	1.04	1.06
4	0.48	0.40	26.9	24.8	11.18	10.96	0.97	0.91
5	0.57	0.51	25.9	24.2			0.84	0.91
6			21.3	20.2	10.97	10.15	1.11	1.10
7			25.2	23.6	6.08	7.58	0.69	0.72
8			25.5	20.4	18.06	22.79	0.99	0.91
9			16.4	16.6	0.46	0.27	1.35	1.49

Subject	Hemoglobin g/dL Centrifugation	Device	Plasma Hematocrit % Centrifugation	Device	Red Blood Cells millions/ uL Centrifugation	Device	White Blood Cells thousands/uL Centrifugation	Device
1	0.27	0.16						
2	0.27	0.19						
3	0.38	0.24						
4	0.25	0.15						
5	0.48	0.13						
6	0.12	0.05	< 0.1	< 0.1	0	0	0.2	0.1
7	0.16	0.08	< 0.1	< 0.1	0	0	0.2	0.2
8	0.22	0.09	< 0.1	< 0.1	0	0.02	0.2	0.2
9	0.33	0.17	< 0.1	< 0.1	0	0	0.2	0.2

Thus, specific embodiments and applications of magnetic separation have been disclosed. It should be apparent to those skilled in the art, however, that many more modifications besides those already described are possible without departing from the inventive concepts herein. The inventive subject matter, therefore, is not to be restricted except in the spirit of the appended claims.

CLAIMS

What is claimed is:

1. A method of separating a sample containing blood cells into a cell containing portion and a substantially cell depleted portion, comprising:
receiving the sample in a vessel;
combining the sample, an additive, and a plurality of particles, each of the additive, the particles and the cells having a substantial binding to another of the additive, the particles and the cells to produce a cell containing network; and
separating the network from the substantially cell depleted portion at least in part using a magnetic force.

2. The method of claim 1 wherein the sample received in the vessel has a volume of more than about 3 ml.

3. The method of claim 1 wherein the sample received in the vessel has a volume of less than about 1 ml.

4. The method of claim 1 wherein the vessel has at least one flexible wall.

5. The method of claim 1 wherein the vessel has multiple samples wells.

6. The method of claim 1 wherein the particles having a mean volume of between about 5×10^{-24} m^3 to about 5×10^{-6} m^3.

7. The method of claim 1 wherein the substantial binding of the particles results at least in part from the particles having a coating.

8. The method of claim 7 wherein the coating comprises an anti-ligand.

9. The method of claim 7 wherein the coating comprises an antibody.

10. The method of claim 7 wherein the coating comprises a polymer.

11. The method of claim 1 wherein the substantial binding of the additive results at least in part from the additive comprising an anti-ligand.

12. The method of claim 1 wherein the network comprises a primary and a secondary antibody, where the primary antibody has a substantial binding to the surface component of the cells, and the secondary antibody has a substantial binding to the primary antibody.

13. The method of claim any of claims 1 - 12 wherein the cells predominantly comprise red blood cells.

14. The method of claim any of claims 1 - 12 wherein the sample includes white blood cells and platelets.

15. The method of any of claims 1 – 12, further comprising measuring PSA.

17 The method of any of claims 1 – 12 wherein at least 70% by volume of the theoretically available cell depleted portion is separated from the network within ten minutes.

18 The method of any of claims 1 – 12 wherein separating the network produces a separation efficiency of at least 70%.

19 The method of any of claims 1 – 12 wherein separating the network produces a separation efficiency of at least 80%.

20 The method of any of claims 1 – 12 wherein separating the network produces a separation efficiency of at least 90%.

21 The method of any of claims 1 – 12 wherein at least 90% by volume of the theoretically available cell depleted portion is separated from the network within ten minutes, with a separation efficiency of at least 95%.

ABSTRACT

A cell containing sample is separated into a cell containing portion and a substantially cell depleted portion, by mixing the sample with particles to produce a cell containing network, and separating the network from the remaining substantially cell depleted portion using a magnetic force. It is contemplated that whole blood may be used as the sample, and that the cell-containing portion largely comprises a network of interlinked red blood cells. It is especially contemplated that separation involves anti-ligands, preferably primary antibodies that bind to a ligand such as antigen on or in red blood cell membranes, and secondary antibodies that bind to the primary antibodies. It is also contemplated that the primary antibodies can be coupled to the surfaces of paramagnetic beads.

FIG. 1A

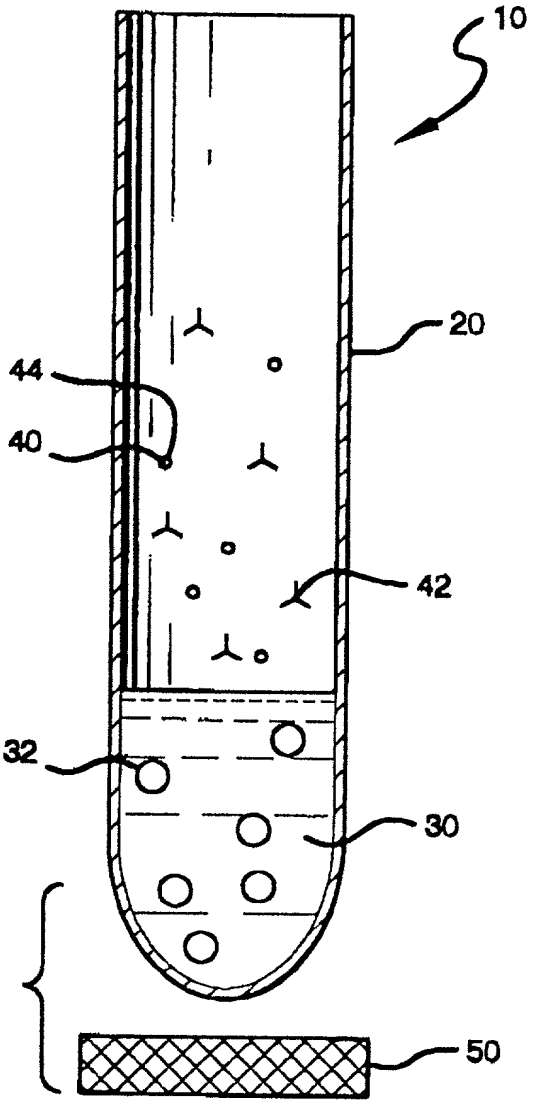


FIG. 1B

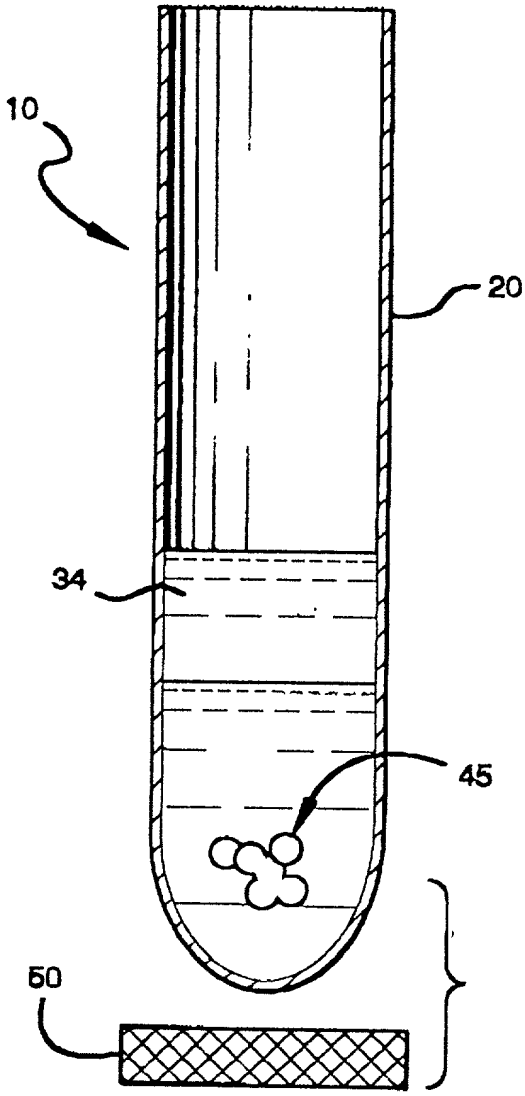
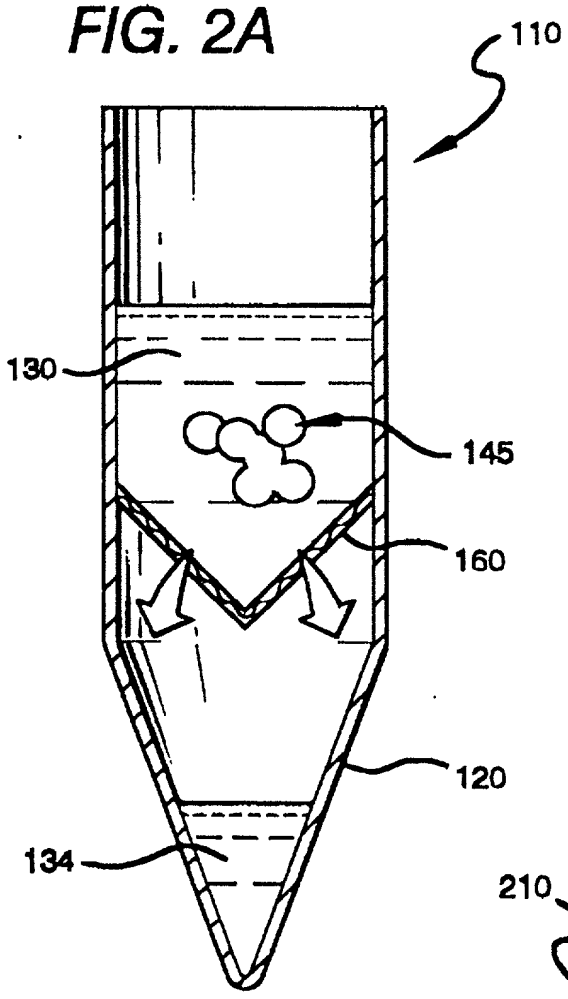


FIG. 2A



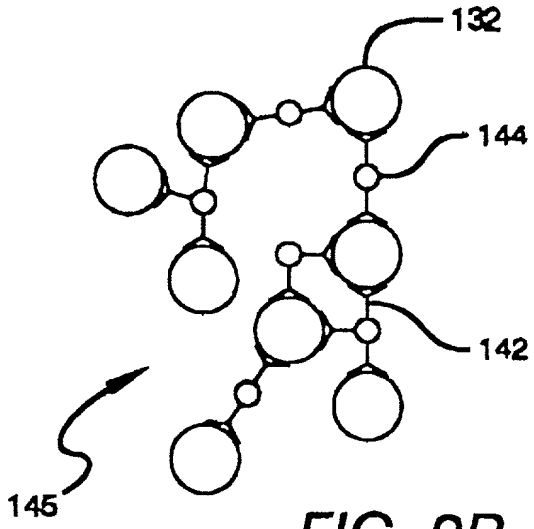


FIG. 2B

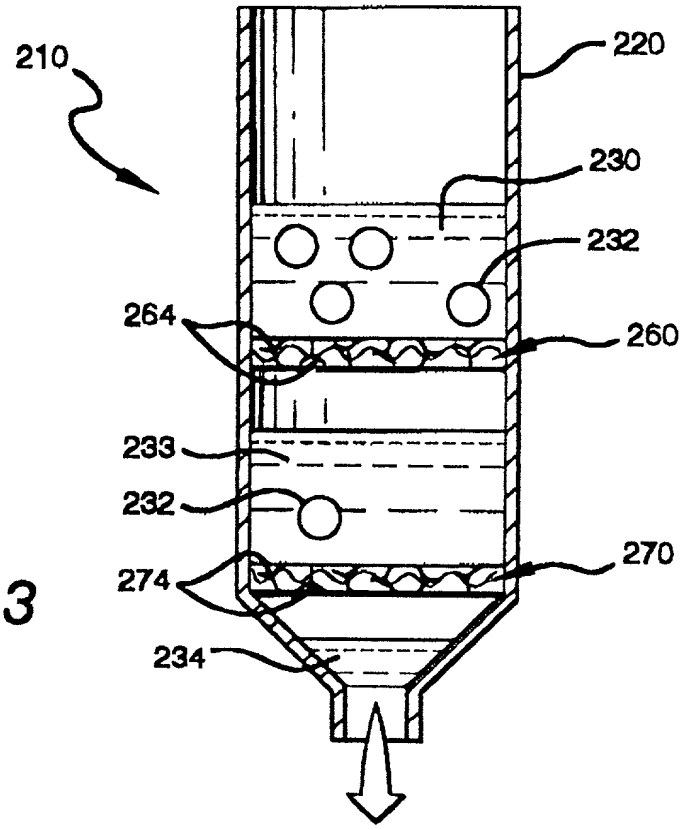


FIG. 3

DECLARATION
AND POWER OF ATTORNEY

UTILITY APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **Methods and Apparatus for Separation of Biological Fluids**, the specification of which

Check One

_X_ is attached hereto.

___ was filed on _____ as Application Serial No. _____.

I have read the applicable statutes and rules reprinted below this declaration which I understand to describe subject matter which is material under 37 CFR 1.56(a).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a). I hereby claim **foreign priority** benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

APPLICATION NO.	COUNTRY	DATE OF FILING	PRIORITY CLAIMED (Y/N)

I hereby claim the benefit under Title 35, United States Code, §120 of **any United States application(s)** listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

Patent
Attorney Docket No.:560.02-US1

APPLICATION NO.	DATE OF FILING	STATUS PATENTED, PENDING OR ABANDONED

I understand that willful false statements and the like are punishable by fine or imprisonment, or both and may jeopardize the validity of the application or any patent issuing thereon. All statements made of my own knowledge are true and all statements made on information and belief are believed to be true. (37 CFR § 1.68)

POWER OF ATTORNEY: As a named inventor, I hereby appoint as my attorneys, with full power of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Robert Douglas Fish, Reg. No. 33,880 and K. David Crockett, Reg. No. 34311, with correspondence directed to Robert D. Fish at Crockett & Fish, 1440 N. Harbor Blvd., Suite 706, Fullerton, CA 92835; Tel: 714-449-2337; Fax: 714-449-2339.

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(Signature(s) should conform to names as presented at 201 et seq. above).